Enzyme-Demethylated Pectinates and Their Gelation[a]

CLAUDE H. HILLS, H. H. MOTTERN,[b] G. C. NUTTING, AND RUDOLPH SPEISER[c]

Eastern Regional Research Laboratory,[d] Philadelphia 18, Pennsylvania

Explanations are offered for the difference in the type of gelation occurring in pectinic acids which have been de-esterified, respectively, by enzymes and by acids.

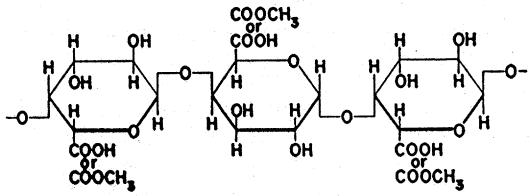
Several years ago this laboratory undertook a study of methods for preparing low-ester pectin from apple pomace as a possible means of increasing the utilization of apple processing wastes. The preparation of low-ester pectins by acid-deesterification had been previously studied by Olsen, et al. *(8)* and by Baker and Goodwin *(1)*. The acid method appeared to possess several disadvantages for commercial usage; it is time consuming, requires acid resistant equipment, and causes some degradation of the product.

The enzyme pectase was considered as a possible catalyst for the deesterification of pectin because the milder conditions of acidity and more rapid rate of reaction would be advantageous in a commercial process. The procedure as developed at our laboratory has been described in previous publications *(5, 7)* and is briefly as follows: A concentrated pectin extract prepared from dried apple pomace is treated with tomato pectase at pH 6.5 and 30° to 40° C. for an hour or less, depending on the concentration of enzyme and the extent of de-esterification desired. The reaction is stopped at the desired point by acidifying the mixture and heating to destroy the enzyme. The product may be isolated by precipitation with ethanol or iso-propanol, and dried in the usual manner.

The pectin complex consists of long straight chain molecules of partially methyl-esterified polygalacturonic acid (Fig. 1) to which are attached at undetermined points side groups of araban and galactan. The molecular weight ranges from relatively low values to approximately 300,000. The araban and galactan contents of a sample may vary from 0 to 30 percent and 0 to 40 percent, respectively, depending upon the source, method of extraction, and subsequent treatment. The methyl ester content (calculated as CH_3O) may vary from approximately 7 to 12 percent for non-deesterified pectins to 0 for pectic acid.

Because of the variable amount of non-galacturonide material present in various preparations of apple pectin, it is more accurate to characterize a preparation by expressing the percent esterification of the polygalacturonide chain than by the weight percent of methoxyl *(4)*. Non-deesterified apple pectin is approximately 70 percent methyl esterified and will form a gel only in the presence of a suitable concentration of sugar and of acid. Pectins of less than 50 percent esterification form the conventional sugar-acid gels, but in addition will gel in the presence of a suitable concentration of a polyvalent cation, such as calcium, in the absence or presence of sugar.

CONSTITUTION AND STRUCTURE OF PECTINIC ACIDS



POLYGALACTURONIDE CHAIN

Degree of esterification	0 to 100%
Araban content	0 to 30%
Galactan content	0 to 40%
Average Molecular Weight	Up to 300,000

FIG. 1. Constitution and structure of pectinic acids. From Speiser, Hills, and Eddy *(15)*.

Thus it is possible to distinguish two different types of pectin gels; (1) hydrogen-bonded gels, such as the usual pectin-sugar-acid jellies; and (2) ionic-bonded gels made with a polyvalent cation such as calcium. The strength of hydrogen-bonded pectin gels depends principally on the molecular weight. The strength of calcium-pectinate gels is influenced also by the mode of deesterification. In general, enzyme-deesterified pectinic acids make weaker gels than do acid-deesterified *(5, 13)*. The present paper will describe some of the properties of enzyme-deesterified pectinic acids and attempt to explain the reasons for their inferior gel strength. Various aspects of this study have been reported in detail in previous publications from this laboratory *(4, 13, 14)*.

Molecular Weight

It is difficult to determine the molecular weight of pectin by viscosity in aqueous solution because of the complex behavior of charged particles in a water solution. To avoid these complications, Schneider and co-workers *(2, 10, 11)* nitrated pectin and measured molecular weights by viscosity in acetone solution, a procedure analogous to that commonly used in cellulose chemistry. It is further possible to fractionate the nitrated pectin and to determine the viscosity and molecular weight of each fraction and to calculate the weight-average and number-average molecular weights.

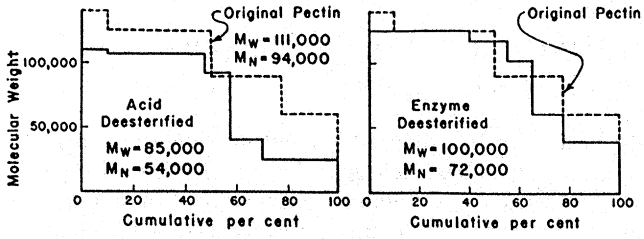
In Fig. 2 are presented histograms showing the molecular weight distribution of a high-ester pectin and two deesterified pectins derived from it by acid and enzyme deesterification, respectively. The total area under each step diagram is equal to the weight average molecular weight. The homogeneity of a sample can be estimated by comparing the weight average molecular weight with the calculated number average molecular weight. It can

[a] Presented before the Eighth Annual I. F. T. Convention, Philadelphia, Pa., June 8, 1948.

[b] Present address: H. J. Heinz Company, Pittsburgh, Pa.

[c] Present address: Ohio State University, Columbus 10, Ohio.

[d] One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture.



Method of Preparation	Degree of Esterification	Methoxyl (CH_3O)	Gel Strength 65% Sugar Jellies	Gel Strength Calcium Pectinate Gels
Acid Deesterified	32%	4.71	20cm.	30cm.
Enzyme Deesterified	36	4.80	47	13
Original Pectin	80	8.87	61	No Gel

FIG. 2. Molecular weight distribution and gelling capacity. From Speiser and Eddy *(14)*.

be seen that the enzyme-deesterified product has undergone slight degradation whereas the acid-deesterified pectinic acid has been degraded to a considerably greater extent. It is also apparent that the strength of the 65 percent sugar jellies prepared from these three pectins is in the same order as their molecular weights. Although the enzyme-deesterified pectinic acid produced a stronger hydrogen-bonded gel than the acid-deesterified sample, it produced a much weaker calcium-pectinate gel. Therefore, the molecular weight is not a sufficient index of the gelling power when comparing acid and enzyme-deesterified pectins.

Non-Galacturonide Materials

It has been shown by Schneider and Bock *(9)* that the characteristic properties of pectin—gelation, film formation, and high viscosity in dilute aqueous solution—derive from the polygalacturonide chain; the non-galacturonide constituents or "ballast materials," chiefly araban and galactan, act mainly as diluents.

Enzyme deesterification causes the removal of very little ballast whereas acid deesterification leads to the removal of ballast at approximately the same rate as the removal of methyl ester groups (Fig. 3). Since the gelling tendency depends principally on the percentage of polygalacturonide material in a given sample, if all other factors are equal, acid-deesterified pectins, gram for gram, will give stronger jellies than enzyme-deesterified pectins. In fact, it has been observed that mild treatment of pectin followed by precipitation and purification results in an improvement of jelly grade *(1)*. It is also possible that during precipitation and purification of acid-treated pectins, low molecular weight fractions would be washed out. This would also tend to increase the grade of the product, but at the expense of reduced yields.

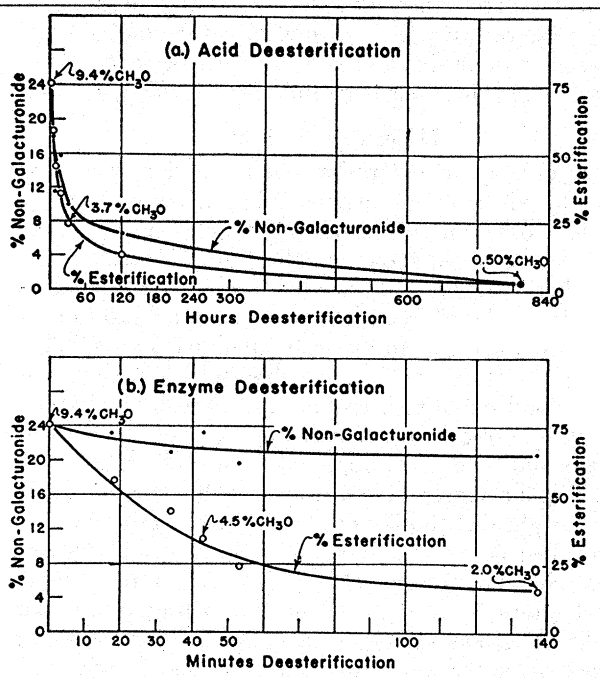


FIG. 3. Rate of removal of methyl ester and non-galacturonide materials during deesterification. From Hills and Speiser *(4)*.

Solubility

Acid- and enzyme-deesterified pectinic acids show marked differences in solubility. Pectic acids or pectinic acids of low degree of esterification prepared by acid deesterification are relatively insoluble in water. Usually a small proportion of the sample will go into solution but the addition of more solvent will not cause additional material to dissolve. This behavior is characteristic of crystalline high polymers such as cellulose, where the alignment of long rigid chains permits the interaction of hydrogen bonding groups to form strongly knit aggregates which require considerable energy to disrupt.

Pectic substances with low degree of esterification and consequently a high concentration of strongly interacting carboxyl groups tend to crystallize upon precipitation or drying and form relatively insoluble aggregates. If there are bulky side groups, or if irregularities of a sufficiently high degree are present in the polymer chains, a lesser extent of crystallization results, and aggregates are formed which admit solvent more freely and dissolve more readily. Enzyme-deesterified pectinic acids, because of their high non-galacturonide content are much more soluble in water than acid-deesterified pectins of corresponding degree of esterification.

Data in Tables 1 and 2 show that acid deesterification, which simultaneously removes ballast and methyl ester groups, effects a greater decrease in solubility than does enzyme, which leaves the ballast content essentially unchanged. The presence of a few percent of alkali metal ions also promotes solubility. Preparations H88F and H89F, containing about 5 percent ash, were readily soluble although almost completely deesterified. Deashing to less than 0.5 percent reduced their solubility considerably. The electrolyte promotes dissociation of the carboxyl groups and so not only decreases the number of strong hydrogen bonds but creates ionized groups which repel one another.

One of the practical factors discouraging the use of very low ester pectinic acids has been their limited solubility. It is now obvious that control of the degree of lateral interaction among the pectinic acid molecules, by choice of enzyme rather than acid deesterification, by incorporating electrolyte or other diluent, and by rapid precipitation and drying, can largely remove this limitation.

Degree of Esterification

It has been demonstrated that the ability of dilute pectin solutions to form calcium-bonded gels, both in

TABLE 1

Solubility of Acid-deesterified Pectinic Acids. Two-gram samples shaken with 100 g. water for 2 hrs. at 27° C. and 100° C.

Sample	Ash	Degree of esterification	Ballast content	Amount dissolved	
				27° C.	100° C.
	%	%	%	%	%
H91	0.25	75	22.2	100	100
H91A	0.22	57	15.0	100	100
H91C	0.20	35	14.5	100	100
H91D	0.40	24	8.2	31	100
H91E	0.53	11	4.7	20	50
H91K	0.37	3	0.9	5	39

Data from Speiser, Copley, and Nutting *(13)*.

TABLE 2

Solubility of Enzyme-deesterified Pectinic Acids. Two-gram samples shaken with 100 g. water for 2 hrs. at 27° C.

Sample	Ash	Degree of esterification	Ballast content	Amount dissolved at 27° C.	Amount of de-ashed material dissolved at 27° C.
	%	%	%	%	%
H91F	0.31	55	18.3	100	
H91G	0.51	45	17.4	100	
H91H	0.55	33	19.7	100	
H91I	0.62	23	16.4	100	100
H91J	1.01	14	16.2	100 (cloudy)	85 (cloudy)
H88F	4.69	3	21.5	100	70 (about)
H89F	4.95	4	19.1	100	74

* De-ashed to 0.5 percent.
Data from Speiser, Copley, and Nutting *(13)*.

the presence and in the absence of sugar, depends on the degree of esterification *(3, 4, 5)*. In 1 percent solutions pectins with greater than 50 percent esterification do not gel with calcium ion alone. The lower the degree of esterification the smaller is the amount of calcium required to form the gel of optimum strength, because of the greater opportunity for forming cross links. The relationship between degree of esterification and calcium requirement for gel formation is shown in Table 3. In this table the percent methoxyl has been used instead of percent esterification since sufficient data were lacking for computation of the latter values.

TABLE 3

Effect of methyl ester content of pectinate on optimum Calcium-pectinate ratio and on gel strengths

CH_3O	Method of deesterification	Gel strength cm. H_2O	Calcium pectinate ratio at optimum
%			
8	Enzyme	16	0.040
7	Enzyme	26	0.020
6	Enzyme	33	0.019
5	Enzyme	37	0.017
7.7	Acid	8	0.073
6.1	Acid	270	0.070
4.9	Acid	180	0.038
4.4	Acid	110	0.029
3.8	Acid	84	0.023

Data from Hills, White, and Baker *(5)*.

It is interesting to note that the enzyme-deesterified pectins increased in gel strength, whereas the acid-deesterified pectins decreased in gel strength, as the methoxyl values decreased. The decrease in gel strength of the acid-deesterified series would be expected since acid causes degradation of the polygalaturonide chain. The increase in gel strength of the enzyme-deesterified series is due to the increase in homogeneity with respect to charge distribution of the molecules within a given sample on continued deesterification.

Charge Distribution

One must remember that the degree of esterification of a pectin sample is an average quantity. Individual molecules in a given preparation may show values considerably higher or lower than the average. Acid and alkali presumably remove ester groups at random, so that each individual molecule has about the same degree of esterification as the average for the entire sample. From our knowledge of the action of enzymes, we would not expect pectase to act in a random manner. To confirm this point, electrophoretic studies were made of a sample of high-ester pectin and of acid- and enzyme-deesterified pectins prepared from it. Since the electrophoretic mobility of a pectin molecule is proportional to charge density, any heterogeneity in degree of esterification should be revealed in the electrophoresis patterns.

Figure 4 shows typical patterns for acid- and enzyme-deesterified pectins and for the pectin from which they were prepared. Both non-deesterified and the acid-deesterified pectin gave sharp boundaries indicating homogeneity of the molecules within each preparation. The broad pattern for the enzyme-deesterified pectin indicates a marked heterogeneity of the molecules with respect to degree of esterification. Ward, Swenson and Owens *(16)* have made similar observations on citrus pectin. They fractionated enzyme-deesterified pectins by chemical means and found marked differences in the degree of esterification of the fractions.

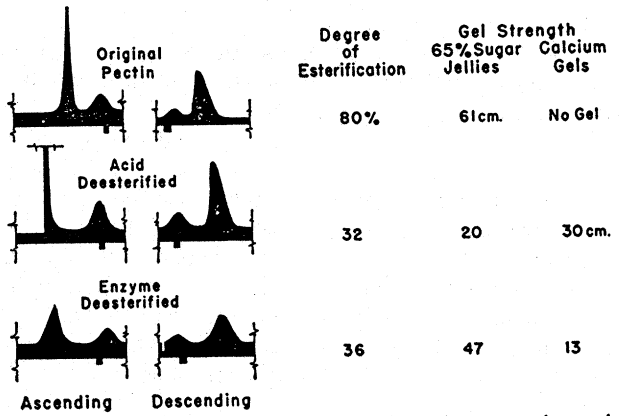


FIG. 4. Electrophoresis patterns for acid and enzyme deesterified pectinic acids. From Hills, Mottern, Nutting, and Speiser *(3)*.

Previously it was pointed out that the amount of calcium ion required for maximum gel strength varied with the degree of esterification. Since most of the molecules in a sample of acid-deesterified pectinic acid would have about the same degree of esterification, a single calcium concentration would be optimum for all the individual molecules and the maximum gel strength would be achieved. However, a single calcium concentration could not be optimum for all the molecules of an enzyme-deesterified pectinic acid. The fraction with

a low degree of esterification would require less calcium than the fraction with a higher degree of esterification. An intermediate calcium concentration would precipitate the former while incompletely cross-linking the latter. It is principally for this reason that enzyme deesterified pectins are inferior in their gel making properties to deesterified pectins produced by acid catalysis.

This explanation was confirmed by the following experiment: Two pectinic acid preparations produced by acid-deesterification, with methoxyl contents of 3.3 and 5.7 percent, gave calcium gels with strength of 88 and 89 cm. The pectinic acids were mixed in equal amounts, giving a methoxy content of 4.5 percent. The calcium gel strength was only 44, or half that of the gel made from each constituent of the mixture. The distribution in degree of esterification was no longer sharp but resembled that of an enzyme-deesterified pectin. A similar mixture made with enzyme-deesterified pectinic acids did not give a correspondingly weakened gel.

This explains the increase in gel strength of the enzyme products with progressive deesterification as observed in Table 3. As the average degree of esterification becomes smaller, the heterogeneity of the molecules inevitably becomes less and the gel strength rises until syneresis reverses the trend.

The possibility should be mentioned that the enzyme may cause another type of non-random demethylation, in which one end of the molecule would be completely deesterified, resembling pectic acid, and the rest of the molecule would be unaltered. Such a condition would also contribute to the observed difference in electrophoretic mobility and gel behavior of the acid and enzyme products. Jansen and MacDonnell *(6)* have accumulated experimental evidence that such hybrid pectic acid-pectin molecules actually result from pectase action, and separate evidence from data by Schultz et al. *(12)* tends to confirm this conclusion.

Summary

The difference in gelation characteristics of enzyme- and acid-deesterified pectinic acids can be explained on the basis of fundamental differences in composition and structure of the two products.

Acid catalysis causes the simultaneous removal of methyl ester groups and non-galacturonide materials. Enzyme deesterification does not affect the non-galacturonide materials, and for that reason the enzyme-prepared pectinic acids are more readily soluble in aqueous solution.

Enzyme-deesterified pectinic acids show broad electrophoretic patterns indicating a marked heterogeneity of the molecules within a given preparation with respect to degree of esterification. Non-deesterified and acid-deesterified pectinic acids are more nearly homogeneous in this respect. This accounts for the inferior gel strength of the enzyme-deesterified product. With increased deesterification, the enzyme product increases in gel strength because of increased homogeneity, but the acid-deesterified product decreases in gel strength because of reduction in molecular weight.

REFERENCES

1. Baker, G. L., and Goodwin, M. W. Delaware Agr. Expt. Sta. *Bull.* 234 (1941).
2. Henglein, F. A., and Schneider, G. *Ber.* 69*B*, 309 (1936).
3. Hills, C. H., Mottern, H. H., Nutting, G. C., and Speiser, R. 108th Meeting of the American Chemical Society, New York, Sept., 1944.
4. Hills, C. H., and Speiser, R. *Science*, 103, 166 (1946).
5. Hills, C. H., White, J. W., and Baker, G. L. *Proc. Inst. Food Tech.*, 1942, p. 47.
6. Jansen, E. F., and MacDonnell, L. R. *Arch. Biochem.*, 8, 97 (1945).
7. Mottern, H. H., and Hills, C. H. *Ind. Eng. Chem.*, 38, 1153 (1946).
8. Olsen, A. G., Stuewer, R. F., Fehlberg, E. R., and Beach, N. M. *Ind. Eng. Chem.*, 31, 1015 (1939).
9. Schneider, G., and Bock, H. *Ber.* 70*B*, 1617 (1937).
10. Schneider, G., and Fritschi, U. *Ber.* 69*B*, 2537 (1936).
11. Schneider, G., and Ziervogel, M. *Ber.* 69*B*, 2530 (1936).
12. Schultz, T. H., Lotzkar, H., Owens, H. S., and Maclay, W. D. *J. Phys. Chem.*, 49, 554 (1945).
13. Speiser, R., Copley, M. J., and Nutting, G. C. *J. Phys. and Colloid Chem.*, 51, 117 (1947).
14. Speiser, R., and Eddy, C. R. *J. A. C. S.*, 68, 287 (1946).
15. Speiser, R., Hills, C. H., and Eddy, C. R. *J. Phys. Chem.*, 49, 328 (1945).
16. Ward, W. H., Swenson, H. A., and Owens, H. S. *J. Phys. Colloid Chem.*, 51, 1137 (1947).